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PRINCIPAL INVESTIGATOR: Rita Nahta, Ph.D.

CONTRACTING ORGANIZATION: Emory University
Atlanta GA 30322

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14. ABSTRACT

Obesity is a major risk factor for breast cancer, and is associated with reduced treatment response and reduced overall survival. The obesity-associated hormones IGF-I and leptin and their receptors, IGF-IR and leptin receptor (Ob-R), are elevated in breast cancer. Co-immunoprecipitation and immunoblotting demonstrated that IGF-IR and Ob-R interact in the breast cancer cell lines MDA-MB-231, MCF7, BT474, and SKBR3. Stimulation of cells with IGF-I promoted Ob-R phosphorylation, which was blocked by IGF-IR kinase inhibition. In addition, IGF-I activated downstream signaling molecules in the leptin receptor and IGF-IR pathways. In contrast to IGF-I, leptin did not induce phosphorylation of IGF-IR, indicating that receptor cross signaling is unidirectional, occurring from IGF-IR to Ob-R. Our results demonstrate for the first time a novel interaction and cross talk between the IGF-I and leptin receptors in human breast cancer cells. Our ongoing studies will examine this cross talk in more detail by determining the biological and molecular effects of inhibition of these growth factor receptors. We will then examine the influence of this cross talk on response to taxane-based chemotherapy.

15. SUBJECT TERMS
Breast cancer, leptin, insulin-like growth factor-I, growth factor receptor signaling

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INTRODUCTION

Obesity is an important risk factor associated with the development and progression of breast cancer (1-7), reduced therapeutic efficacy, and higher mortality rates among breast cancer patients (8-11). The obesity-associated hormones insulin-like growth factor-I (IGF-I) and leptin are found at high levels in breast cancer patients (12-15), and their receptors, IGF-IR and Ob-R (leptin receptor is also known as obesity receptor), are overexpressed in a majority of breast cancers (15-17). Increased expression of leptin and Ob-R correlate with increased risk for distant metastasis and reduced overall survival in breast cancer patients (15). Leptin induces proliferation of breast cancer cells via activation of STAT3 (18,19), a transcriptional activator of the anti-apoptotic protein Bcl-2 (20,21). STAT3-dependent overexpression of Bcl-2 was associated with resistance to the chemotherapeutic agent paclitaxel in breast cancer cells (21). We propose that IGF-IR and the leptin receptor interact, and that IGF-IR and leptin induce phosphorylation of Ob-R, activating STAT3 and upregulating Bcl-2, which in turn results in taxane resistance. Our hypothesis is that high levels of leptin and IGF-I increase Ob-R signaling, contributing to taxane resistance in breast cancer. Our long-term goal is to establish markers of Ob-R signaling as predictors of taxane response. The rationale is that these markers of Ob-R signaling, including serum levels of leptin and IGF-I, and tissue levels of phosphorylated Ob-R, STAT3 and Bcl-2, can be used (1) to identify patients most likely to respond to taxanes, and (2) as therapeutic targets to improve response rates to taxanes in the treatment of breast cancer.

BODY

Task 1 Apply nanotechnology-based methods for visualization of IGF-IR and leptin receptor (Ob-R) in real time.

This aim is being done in collaboration with Dr. Konstantin Sokolov at MD Anderson. His team has had success with labeling antibodies with gold or silver nanoparticles. We will continue to work on the best way to label IGF-IR and Ob-R antibodies and to image cells exposed to these antibodies in the upcoming year.

Task 2 Demonstrate that IGF-I activates the leptin receptor via IGF-IR crosstalk.

This aim was the focus of a publication currently *in press* (**please see attached appendix for figures**). Results of that publication are summarized below.

The human breast cancer lines MDA-MB-231 (MDA231), MCF7, BT474, and SKBR3 were examined for expression of the IGF-I receptor (IGF-IR) and leptin receptor (Ob-R). Immunoblotting of total protein lysates (Figure 1A, see appendix) demonstrated that the two major isoforms of Ob-R, called Ob-Rb (longer isoform) and Ob-Rt (shorter isoform) are expressed at similar levels in all cell lines (Figure 1B, see appendix). IGF-IR is expressed at higher levels in MCF7 and BT474 cells versus SKBR3 and MDA231 cells, with highest levels observed in MCF7 cells (Figure 1B, see appendix).

Immunoprecipitation of Ob-R with subsequent immunoblotting for IGF-IR showed that Ob-Rb and Ob-Rt are both pulled down with IGF-IR in all four cell lines (Figure 2A, see appendix). Conversely, IGF-IR immunoprecipitation pulled down Ob-Rb and Ob-Rt in each cell line, with preferential interaction observed with the shorter isoform of Ob-R in MCF7, BT474, and SKBR3 cells (Figure 2B, see appendix). Quantitation showed that IGF-IR was pulled down with Ob-R to a similar extent in all four lines (Figure 2C, see appendix). Total Ob-R was pulled down with IGF-IR in all four lines; however, higher levels of Ob-R interacting with IGF-IR was observed in MCF7 cells (Figure 2C, see appendix), likely due to the higher expression level of total IGF-IR in these cells (Figure 1B, see appendix). Negative controls in which cell lysates were immunoprecipitated with rabbit IgG confirmed that IGF-IR and Ob-R were not pulled down (Figure 2D, see appendix). In addition, since IGF-IR has been shown to interact with insulin receptor (Ins-R) (21), we blotted IGF-IR immunoprecipitates for Ins-R as a positive control (Figure 2D, see appendix). Ins-R was pulled down with IGF-IR in all four lines. Finally, another tyrosine kinase receptor, EGFR, was immunoprecipitated and blotted for Ob-R in all lines (Figure 2D, see appendix). Collectively, the results of these immunoprecipitation experiments indicate that the insulin-like growth factor-I receptor and leptin receptor interact in human breast cancer cells.

To determine the effect of IGF-IR/leptin receptor interaction on receptor signaling, MCF7 cells were serum-starved overnight, and then stimulated with IGF-I (100 ng/mL) for up to one hour. IGF-IR phosphorylation was induced within 5 min (Figure 3A, see appendix), while total IGF-IR levels were unaltered. Importantly, phosphorylation of Ob-R was also induced within 5 min of IGF-I exposure, suggesting potential cross signaling from IGF-IR to leptin receptor. Similarly, in BT474 cells (Figure 3B, see appendix) and MDA231 cells (Figure 3C, see appendix), IGF-I stimulation induced phosphorylation of both IGF-IR and Ob-R within 5 min, without affecting total levels of either receptor. To determine if IGF-I stimulates phosphorylation of the leptin receptor via the IGF-IR kinase, MCF7 cells were treated with the IGF-IR kinase inhibitor I-OMe-AG538 and stimulated with IGF-I (Figure 3D, see appendix). Immunoblotting demonstrated that inhibition of IGF-IR kinase blocked IGF-I-stimulated phosphorylation of leptin receptor. Thus, IGF-I cross signals to the leptin receptor via the IGF-IR kinase.

Having established that IGF-IR stimulates phosphorylation of the leptin receptor, we examined IGF-I-mediated effects on downstream receptor signaling. MCF7 cells were stimulated with IGF-I and immunoblotted for phosphorylated and total JAK2 and STAT3 (Figure 4A, see appendix) and for phosphorylated and total Akt, ERK1/2, and p38MAPK (Figure 4B, see appendix). Significant phosphorylation of JAK2 and STAT3 was observed in response to IGF-I within 5 min. IGF-I also activated the PI3K pathway as shown by phosphorylation of Akt. Phosphorylation of ERK1/2 and p38 MAPK was rapidly activated but transient versus other signaling pathways. Collectively, these results support the concept that IGF-I cross activates the leptin receptor signaling pathway, although the signaling molecules examined are downstream of multiple growth factor receptors, and thus do not strictly confirm activation of leptin receptor signaling. However, as leptin receptor phosphorylation was induced by IGF-I and blocked by IGF-IR kinase inhibitor on tyrosine 1141, which is the phosphorylation site that binds STAT3 and activates downstream signaling, our results strongly suggest that IGF-IR induces activation of the leptin receptor.

We next examined whether cross talk occurs in the opposite direction, i.e. from the leptin receptor to IGF-IR. MCF7 cells were serum starved and stimulated with leptin (1000 ng/mL) for up to 6 hours. Leptin induced phosphorylation of leptin receptor within 5 min (Figure 5A, see appendix). However, phosphorylation of IGF-IR at either tyrosine 1131 or tyrosine 1135 and1136 was not stimulated by leptin at these time points up to 6 hours, nor was it stimulated at shorter time point increments or longer time points up to 24 hours or with lower doses of leptin (not shown). As a positive control, IGF-I stimulated phosphorylation of IGF-IR as expected and also induced phosphorylation of leptin receptor as previously observed (Figure 3, see appendix). Similarly, BT474 cells stimulated with leptin showed phosphorylation of leptin receptor but not of IGF-IR at either of the three sites examined (Tyrosine 1131, 1135, 1136) (Figure 5B, see appendix). Thus, our results suggest a unidirectional cross talk from the IGF-I receptor to the leptin receptor in breast cancer cells.

In the current funding period, we will examine the effects of this cross talk on Bcl-2 as described in the original proposal.

Task 3 Demonstrate that Ob-R signaling activated by leptin or IGF-I contributes to taxane resistance.

We reported in the last annual report that breast cancer cells treated with leptin and taxane showed very little change in DNA fragmentation. While the past funding period focused heavily on aim 2, resulting in a publication, the upcoming funding period will focus on this aim 3. We will test additional doses and time points, as well as other assays (flow cytometry, trypan blue exclusion), as described in the original proposal. We are also working in collaboration with other investigators at MD Anderson to examine association of leptin receptor signaling molecules with resistance to taxanes in breast cancer patients, as described for years 2 and 3 of the proposal.

KEY RESEARCH ACCOMPLISHMENTS

- Discovery that IGF-IR and leptin receptor interact
- Discovery that IGF-I promotes leptin receptor phosphorylation
- Discovery that IGF-IR kinase inhibition blocks signaling to leptin receptor
- Discovery that cross talk is unidirectional

REPORTABLE OUTCOMES

- *In press* publication (see appendix)
- Invited speaker, DoD BCRP 2008 Era of Hope meeting to present the results of this grant at an oral symposium
- Invited to present poster of this work also at DoD BCRP 2008 Era of Hope meeting
- Started own lab as a tenure track faculty member at Emory University

- ❖ Selected as a Georgia Cancer Coalition Scholar based on funding and publication history

CONCLUSION

We made the following novel discoveries. (1) The IGF-I and leptin receptors interact in human breast cancer cells. (2) Cross signaling occurs from IGF-IR to Ob-R in breast cancer. IGF-I stimulation induces phosphorylation and activation of Ob-R, while IGF-IR kinase inhibition blocks IGF-I-mediated Ob-R activation. Downstream signaling molecules JAK2, STAT3, Akt, and ERK1/2, all of which are functional in the leptin and IGF-IR pathways as well as in multiple other signaling pathways, were activated by IGF-I stimulation. (3) Cross talk is unidirectional, as leptin does not activate IGF-IR. Thus, leptin is not likely to affect IGF-IR oncogenic function in breast cancer. However, since IGF-IR cross talks to Ob-R, it is feasible that Ob-R may contribute to IGF-IR molecular or biological effects, and is worthy of further study. Thus, we have identified a novel receptor interaction and unidirectional cross talk involving the IGF-IR and leptin receptor. Our future experiments will closely examine the effect of leptin/IGF-I cross talk on Bcl2 and taxane resistance. The results so far are significant in that they suggest interaction between two growth factor receptor signaling pathways that play an important part in breast cancer biology. The implication is that targeting one receptor may not be enough; dual targeting against IGF-IR and leptin receptor may be more beneficial and should be examined. In addition, determining the role of these receptor pathways in taxane responsiveness may ultimately contribute to being able to predict which patients will respond to this commonly used chemotherapeutic agent.

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APPENDICES

Please see the attached Curriculum Vitae.
Please see attached publication in press.

SUPPORTING DATA

N/A

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Rita Nahta	Assistant Professor
eRA COMMONS USER NAME (credential, e.g., agency login) rnahta	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
UNC, Chapel Hill, N.C.	B.S.	1994	Chemistry
Duke University, Durham, N.C.	Ph.D.	2000	Field: Molecular Cancer Biology (Department: Pathology)
Harvard Medical School	N/A	2000-2002	Postdoctoral
M. D. Anderson Cancer Center	N/A	2002-2004	Postdoctoral

A. Positions and Honors. List in chronological order previous positions, concluding with your present position. List any honors. Include present membership on any Federal Government public advisory committee.

Positions

1990-1994 (summers only) Lab Technician, The Virkler Company, Charlotte, NC, Supervisor: Peter Hauser, Ph.D.
1995-2000 Ph.D. Candidate, Department of Pathology and Molecular Medicine, Duke University, Durham, NC, Mentors: J. Dirk Iglehart, M.D. and Jeffrey R. Marks, Ph.D.
2000-2002 Postdoctoral Fellow, Department of Medicine and Tumor Biology, Harvard Medical School and Massachusetts General Hospital Cancer Center, Boston, MA, Mentor: Emmett V. Schmidt, M.D., Ph.D.
2002-2004 Postdoctoral Fellow, Department of Breast Medical Oncology, M. D. Anderson Cancer Center, Houston, TX, Mentor: Francisco J. Esteva, M.D., Ph.D.
2004-2/2007 Instructor, Department of Breast Medical Oncology, M. D. Anderson Cancer Center, Houston, TX
9/2006-present Invited Managing Editor, Frontiers in Bioscience
2/2007-present Assistant Professor, tenure track, joint appointment: Department of Pharmacology and Department of Hematology/Oncology, School of Medicine and Winship Cancer Institute, Emory University
9/2007-present Assistant Professor, Molecular Systems Pharmacology Program, Graduate Division of Biological and Biomedical Sciences, Emory University

Honors

2004 AACR-Amgen, Inc. Fellowship Award in Clinical/Translational Cancer Research
2004 M. D. Anderson Cancer Center Odyssey Special Fellowship Award
2004 Concept Award, Department of Defense Breast Cancer Research Program
2005 IDEA Award, Department of Defense Breast Cancer Research Program
2006 Howard Temin K01 Award, National Cancer Institute
2007 Distinguished Cancer Scholar Award, Georgia Cancer Coalition

B. Selected peer-reviewed publications (in chronological order).

1. Dong Q, Johnson SP, Colvin OM, Bullock N, Kilborn C, Runyon G, Sullivan DM, Easton J, Bigner DD, Nahta R, Marks JR, Modrich P, and Friedman HS. Multiple DNA repair mechanisms and alkylator resistance in the human medulloblastoma cell line D-283 Med (4-HCR). Cancer Chemother Pharmacol 43:73-79, 1999.
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C. Research Support.

Current Support

National Cancer Institute K01CA118174 8/1/2006-7/31/2011
Role: PI
Title: "HER-2/IGF-IR cross-talk and Herceptin resistance"
Aims of this study are to investigate interaction and cross talk between HER2 and IGF-IR, downstream signaling and downregulation of p27kip1, and targeting IGF-IR in Herceptin resistance.

U. S. Department of Defense (DOD) W81XWH 0610452 3/20/2006-3/19/2009
Role: PI
Title: “Crosstalk between leptin receptor and IGF-IR in breast cancer: a potential mediator of chemoresistance”
Aims of this study are to investigate potential interaction and cross talk between the IGF-I receptor and the leptin receptor and effects of leptin signaling on resistance to taxane chemotherapy.

Georgia Cancer Coalition 7/01/2007-6/30/2012
Role: PI
Title: Distinguished Cancer Scholar Program

Completed Support
U. S. Department of Defense (DOD) W81XWH 0510419 6/15/05-6/14/06
Role: PI
Title: “BTG1, a novel mediator of chemosensitivity in breast cancer”
Aims of this study were to examine regulation of BTG1 by Bcl2 and to demonstrate that BTG1 sensitizes breast cancer cells to chemotherapy.

Institutional Research Grant 9/1/05-8/31/06
Role: Co-PI
M. D. Anderson Cancer Center
Title: “Mechanisms of p27kip1 downregulation in trastuzumab-resistant breast cancer cells”
Aims were to examine mechanisms of p27kip1 downregulation focusing on IGF-I signaling.

AACR-Amgen, Inc. Postdoctoral Fellowship in Clinical/Translational Cancer Research
7/1/04-6/30/05
Role: PI
American Association for Cancer Research and Amgen, Inc.
Title: “p27kip1 as a therapeutic target in trastuzumab-resistant breast cancer”
Aims of this study were to examine mechanisms of p27kip1 downregulation in trastuzumab-resistant breast cancer and to correlate immunostaining of p27kip1 with response to trastuzumab in breast cancer tissues.

M. D. Anderson Cancer Center Odyssey Special Fellowship
Role: PI
2004
Title: “Mechanisms of trastuzumab resistance in breast cancer”
Aims were to characterize trastuzumab-resistant breast cancer cells using microarray and to determine the role of IGF-I signaling in trastuzumab resistance.

A Novel Unidirectional Cross-Talk from the Insulin-Like Growth Factor-I Receptor to Leptin Receptor in Human Breast Cancer Cells

Tuba Ozbay[1,3] and Rita Nahta[1,2,3,4]

Departments of [1]Pharmacology and [2]Hematology/Oncology, School of Medicine, [3]Winship Cancer Institute, and [4]Molecular and Systems Pharmacology Program, Graduate Division of Biological and Biomedical Sciences, Emory University, Atlanta, Georgia

Abstract

Obesity is a major risk factor for the development and progression of breast cancer. Increased circulating levels of the obesity-associated hormones leptin and insulin-like growth factor-I (IGF-I) and overexpression of the leptin receptor (Ob-R) and IGF-I receptor (IGF-IR) have been detected in a majority of breast cancer cases and during obesity. Due to correlations between increased leptin, Ob-R, IGF-I, and IGF-IR in breast cancer, we hypothesized that molecular interactions may exist between these two signaling pathways. Coimmunoprecipitation and immunoblotting showed that IGF-IR and Ob-R interact in the breast cancer cell lines MDA-MB-231, MCF7, BT474, and SKBR3. Stimulation of cells with IGF-I promoted Ob-R phosphorylation, which was blocked by IGF-IR kinase inhibition. In addition, IGF-I activated downstream signaling molecules in the leptin receptor and IGF-IR pathways. In contrast to IGF-I, leptin did not induce phosphorylation of IGF-IR, indicating that receptor cross-signaling is unidirectional, occurring from IGF-IR to Ob-R. Our results show, for the first time, a novel interaction and cross-talk between the IGF-I and leptin receptors in human breast cancer cells.
(Mol Cancer Res 2008;6(6):1–7)

Background

Obesity is an important and manageable risk factor for the development and progression of postmenopausal breast cancer (1). Increased body weight and body mass index are associated with reduced disease-free and overall survival and poorer therapeutic response rates in breast cancer patients, regardless of menopausal status or age (2). Although the exact molecular mechanisms by which obesity influences cancer biology are unknown, there is evidence suggesting that increased production and secretion of adipocyte-derived growth factors and hormones contributes to cellular transformation and tumorigenesis (3, 4). The obesity-associated hormones leptin and insulin-like growth factor-I (IGF-I) have been independently implicated in the connection between obesity and breast cancer (5).

Leptin, a product of the obese (*ob*) gene, is an adipocytokine that regulates appetite, bone formation, reproduction, cellular proliferation, and angiogenesis (6). Because of the strong association between human obesity and elevated levels of circulating leptin, this hormone has been widely studied in the fields of nutrition and weight management (7). More recently, however, leptin has emerged as a potential factor contributing to mammary tumorigenesis. *In vitro* studies showed that leptin stimulates the growth, survival, and transformation of breast cancer cells (5), primarily by activating the Janus-activated kinase (JAK)/signal transducers and activators of transcription (STAT) signaling pathway (8, 9) and the phosphoinositol-3-kinase/Akt and mitogen-activated protein kinase (MAPK) pathways (10). Leptin induces cell cycle progression by up-regulating cyclin D1 expression and cyclin-dependent kinase 2 activity, as well as by inactivating the retinoblastoma growth suppressing protein (11). Importantly, leptin and its receptor (Ob-R) were found to be overexpressed in a majority of breast cancer tissues, especially in high-grade tumors, but absent or expressed at very low levels in normal mammary epithelium or benign tumors (5, 12). In addition, leptin-deficient mice have a decreased incidence of spontaneous and oncogene-induced mammary tumors (13). Thus, leptin signaling seems to play an important role in breast cancer biology.

Similar to leptin, increased levels of IGF-I and its receptor are detected in sera and primary tumors of breast cancer patients (14, 15), and transgenic overexpression of IGF-I receptor (IGF-IR) has been shown to induce mammary tumor formation (16). IGF-I is an important endocrine, paracrine, and autocrine regulator of breast epithelial cell growth. Increased signaling through the IGF-IR results in increased cellular proliferation, mitogenesis, and survival and decreased apoptosis, causing resistance to numerous antineoplastic agents (14, 17). For these reasons, the IGF-IR has become an important therapeutic target for drug discovery in breast oncology (17).

Cross-talk between different growth factor receptor families is frequently observed in tumors. This mechanism allows cancer cells to enhance downstream signaling resulting in greatly increased proliferation, mitogenesis, and cell survival.

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The IGF-IR has been shown to interact and cross-talk with multiple receptors, including the epidermal growth factor receptor (EGFR; ref. 18), HER2 (19), platelet-derived growth factor receptor (20), and the estrogen receptor (14). Due to the correlations between elevated levels of leptin, IGF-I, and their associated receptors with obesity and breast cancer, we hypothesized that interactions and/or cross-talk may occur between these two signaling pathways.

Results

IGF-IR and Leptin Receptor Interact in Human Breast Cancer Cells

The human breast cancer lines MDA-MB-231 (MDA231), MCF7, BT474, and SKBR3 were examined for expression of the IGF-IR and leptin receptor (Ob-R). Immunoblotting of total protein lysates (Fig. 1A) showed that the two major isoforms of Ob-R, called Ob-Rb (longer isoform) and Ob-Rt (shorter isoform), are expressed at similar levels in all cell lines (Fig. 1B). IGF-IR is expressed at higher levels in MCF7 and BT474 cells versus SKBR3 and MDA231 cells, with highest levels observed in MCF7 cells (Fig. 1B).

Immunoprecipitation of Ob-R with subsequent immunoblotting for IGF-IR showed that Ob-Rb and Ob-Rt are both pulled down with IGF-IR in all four cell lines (Fig. 2A). Conversely, IGF-IR immunoprecipitation pulled down Ob-Rb and Ob-Rt in each cell line, with preferential interaction observed with the shorter isoform of Ob-R in MCF7, BT474, and SKBR3 cells (Fig. 2B). Quantitation showed that IGF-IR was pulled down with Ob-R to a similar extent in all four lines (Fig. 2C). Total Ob-R was pulled down with IGF-IR in all four lines; however, higher levels of Ob-R interacting with IGF-IR was observed in MCF7 cells (Fig. 2C), likely due to the higher expression level of total IGF-IR in these cells (Fig. 1B). Negative controls in which cell lysates were immunoprecipitated with rabbit IgG confirmed that IGF-IR and Ob-R were not pulled down (Fig. 2D). In addition, because IGF-IR has been shown to interact with insulin receptor (21), we blotted IGF-IR immunoprecipitates for insulin receptor as a positive control (Fig. 2D). Insulin receptor was pulled down with IGF-IR in all four lines. Finally, another tyrosine kinase receptor, EGFR, was immunoprecipitated and blotted for Ob-R in all lines (Fig. 2D). Collectively, the results of these immunoprecipitation experiments indicate that the IGF-IR and leptin receptor interact in human breast cancer cells.

IGF-IR Cross-Signals to the Leptin Receptor

To determine the effect of IGF-IR/leptin receptor interaction on receptor signaling, MCF7 cells were serum-starved overnight and then stimulated with IGF-I (100 ng/mL) for up to 1 hour. IGF-IR phosphorylation was induced within 5 minutes (Fig. 3A), while total IGF-IR levels were unaltered. Importantly, phosphorylation of Ob-R was also induced within 5 minutes of IGF-I exposure, suggesting potential cross-signaling from IGF-IR to leptin receptor. Similarly, in BT474 cells (Fig. 3B) and MDA231 cells (Fig. 3C), IGF-I stimulation induced phosphorylation of both IGF-IR and Ob-R within 5 minutes, without affecting total levels of either receptor. To determine if IGF-I stimulates phosphorylation of the leptin receptor via the IGF-IR kinase, MCF7 cells were treated with the IGF-IR kinase inhibitor I-OMe-AG538 and stimulated with IGF-I (Fig. 3D). Immunoblotting showed that inhibition of IGF-IR kinase blocked IGF-I–stimulated phosphorylation of leptin receptor. Thus, IGF-I cross-signals to the leptin receptor via the IGF-IR kinase.

Having established that IGF-IR stimulates phosphorylation of the leptin receptor, we examined IGF-I–mediated effects on downstream receptor signaling. MCF7 cells were stimulated with IGF-I and immunoblotted for phosphorylated and total JAK2 and STAT3 (Fig. 4A) and for phosphorylated and total Akt, extracellular signal-regulated kinase 1/2 (ERK1/2), and p38 MAPK (Fig. 4B). Significant phosphorylation of JAK2 and STAT3 was observed in response to IGF-I within 5 minutes. IGF-I also activated the phosphoinositol-3-kinase pathway, as shown by phosphorylation of Akt. Phosphorylation of ERK1/2 and p38 MAPK was rapidly activated by

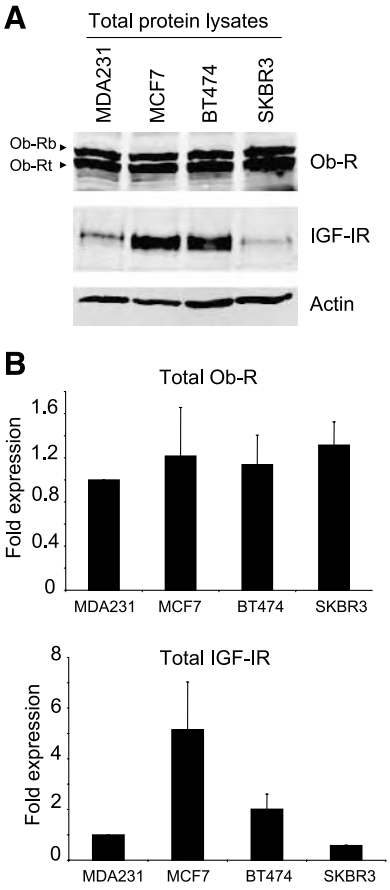

FIGURE 1. Expression of IGF-IR and Ob-R in breast cancer lines. The breast cancer lines MDA-MB-231 (MDA231), MCF7, BT474, and SKBR3 were lysed for total protein. **A.** Total protein lysates were immunoblotted for Ob-R using the H-300 polyclonal antibody, which recognizes both the long Ob-Rb isoform and the shorter Ob-Rt isoform of the leptin receptor. Immunoblotting was also done for total IGF-IR and for actin as a loading control. **B.** Bands on immunoblots were quantitated using NIH ImageJ and are expressed relative to expression levels in MDA231 cells (*lane 1*). Error bars, SD between three independent experiments. Total Ob-R levels were similar among the four lines; IGF-IR was expressed at the highest level in MCF7 cells, with BT474 cells showing moderate expression compared with the other two lines which expressed the lowest levels of IGF-IR.

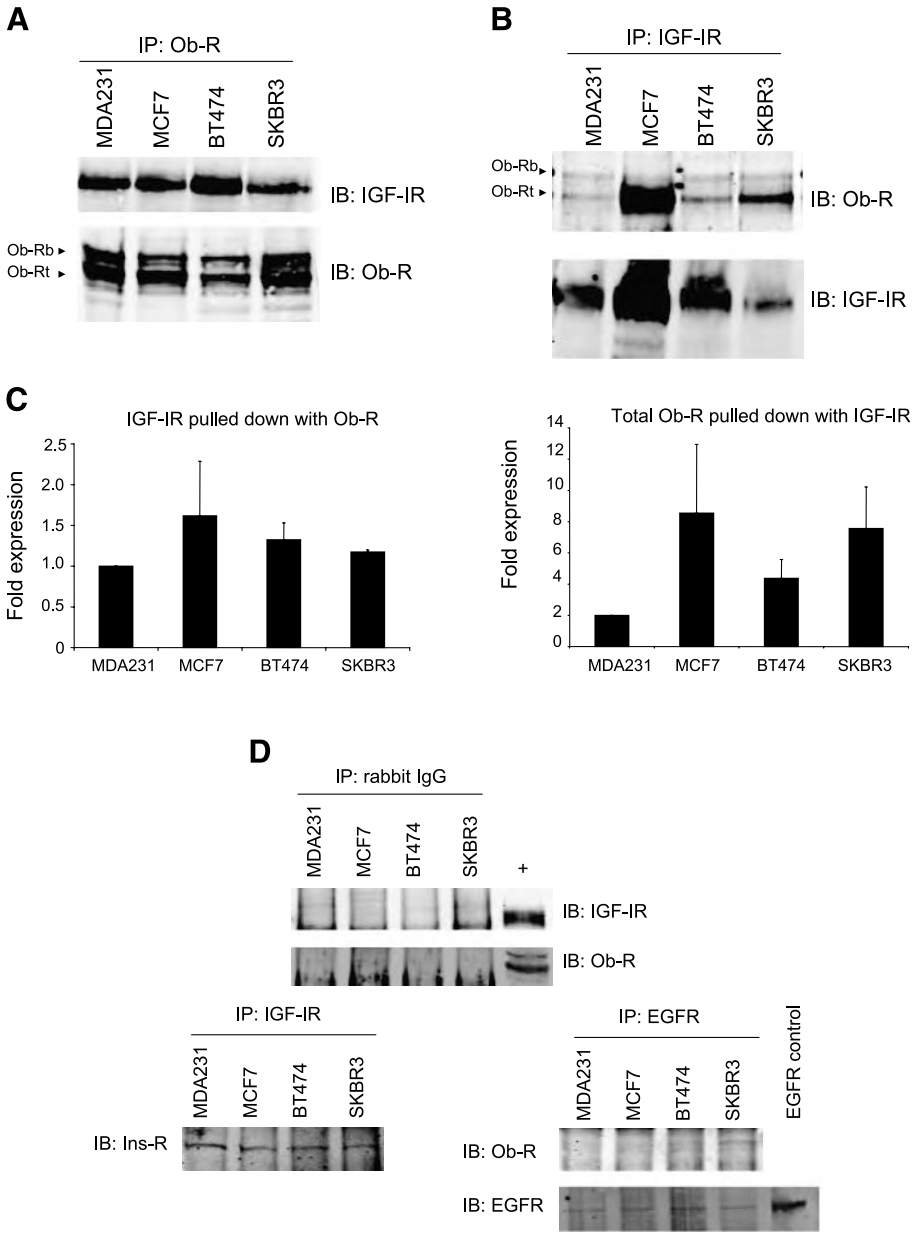

FIGURE 2. Interaction between IGF-IR and Ob-R in breast cancer. The breast cancer lines MDA-MB-231 (MDA231), MCF7, BT474, and SKBR3 were lysed for total protein. Ob-R (**A**) and IGF-IR (**B**) were immunoprecipitated (*IP*; 1 μg of antibody) from total protein extracts (200 μg) and immunoblotted (*IB*) to detect IGF-IR and Ob-R. Ob-R immunoprecipitation pulled down IGF-IR; conversely, IGF-IR immunoprecipitation pulled down Ob-R. **C.** Quantitation of immunoprecipitated experiments. Error bars, SD between three independent experiments. Values were normalized to the MDA231 cells (*lane 1*). **D.** Cell lysates were immunoprecipitated using 1 μg rabbit IgG and immunoblotted for IGF-IR and Ob-R as a negative control. On IGF-IR blot, total lysate from MCF7 cells is included as a positive control (+) for the antibody; on Ob-R blot, lysate from COLO320DM cells was purchased as a positive control (+) for the H-300 antibody from Santa Cruz. As a positive immunoprecipitated control, cell lysates were immunoprecipitated with IGF-IR antibody and blotted for insulin receptor, which is known to interact with IGF-IR. EGFR tyrosine kinase receptor was also immunoprecipitated and blotted for Ob-R, with MDA231 total cell lysate added as a positive control for EGFR. Our results show that the IGF-IR and leptin receptor form a protein complex in breast cancer cells.

transient versus other signaling pathways. Collectively, these results support the concept that IGF-I cross-activates the leptin receptor signaling pathway, although the signaling molecules examined are downstream of multiple growth factor receptors and, thus, do not strictly confirm activation of leptin receptor signaling. However, as leptin receptor phosphorylation was induced by IGF-I and blocked by IGF-IR kinase inhibitor on Tyr^{1141}, which is the phosphorylation site that binds STAT3 and activates downstream signaling, our results strongly suggest that IGF-IR induces activation of the leptin receptor.

IGF-IR/Leptin Receptor Cross-Talk is Unidirectional

We next examined whether cross-talk occurs in the opposite direction, i.e., from the leptin receptor to IGF-IR. MCF7 cells were serum starved and stimulated with leptin (1,000 ng/mL) for up to 6 hours. Leptin induced phosphorylation of leptin receptor within 5 minutes (Fig. 5A). However, phosphorylation of IGF-IR at either Tyr^{1131} or $Tyr^{1135/1136}$ was not stimulated by leptin at these time points of up to 6 hours nor was it stimulated at shorter time point increments or longer time points of up to 24 hours or with lower doses of leptin (not shown). As a positive control, IGF-I stimulated phosphorylation of IGF-IR as expected and also induced phosphorylation of leptin receptor as previously observed (Fig. 3). Similarly, BT474 cells stimulated with leptin showed phosphorylation of leptin receptor but not of IGF-IR at either of the three sites examined (Tyr^{1131}, Tyr^{1135}, and Tyr^{1136}; Fig. 5B). Thus, our results suggest a unidirectional cross-talk from the IGF-IR to the leptin receptor in breast cancer cells.

Discussion

Epidemiologic studies estimate that obesity increases the risk of breast cancer by up to 50% (3). The molecular mechanisms guiding obesity-associated breast cancer are not well understood, but are likely to involve an increased production and secretion of obesity-associated hormones (22). IGF-I and leptin are capable of regulating mammary tissue growth at multiple levels (5). Both hormones are secreted by abdominal adipocytes, resulting in endocrine effects on various tissues, including the breast. Paracrine growth stimulatory effects occur via IGF-I and leptin released by the adipocyte component of stroma surrounding breast epithelial cells or existing breast tumor cells. In addition, an autocrine signaling component is present as breast cancer cells themselves produce and secrete IGF-I and leptin and express cell surface receptors for both ligands. Thus, IGF-I and leptin represent a molecular link between adipose tissue and mammary tissue.

The IGF-IR and Ob-R signaling pathways have each been independently implicated in the development and progression of breast cancer. High circulating levels of IGF-I have been associated with an increased risk of developing breast cancer, and patients with existing breast cancer expressed high serum levels of IGF-I (17). In addition, transgenic mouse models overexpressing IGF-I, IGF-II, or IGF-IR showed an increased incidence of mammary tumor formation (16, 17, 23, 24). Conversely, liver-specific depletion of IGF-I caused reduced circulating levels of IGF-I in mice, resulting in diminished IGF-I endocrine effects on mammary tissue and, ultimately, reduced incidence of breast tumors (25). Similar to the IGF-I signaling pathway, leptin signaling has been associated with breast cancer. Leptin and its receptor were shown by immunohistochemistry to be overexpressed in primary and metastatic breast cancers relative to noncancer tissues (5). Expression of both leptin and Ob-R was most abundant among high-grade tumors, supporting a role for this pathway in breast cancer progression. In addition, *in vivo* models showed that whereas mice that overexpress transforming growth factor-α developed mammary tumors, leptin-deficient transforming growth factor-α

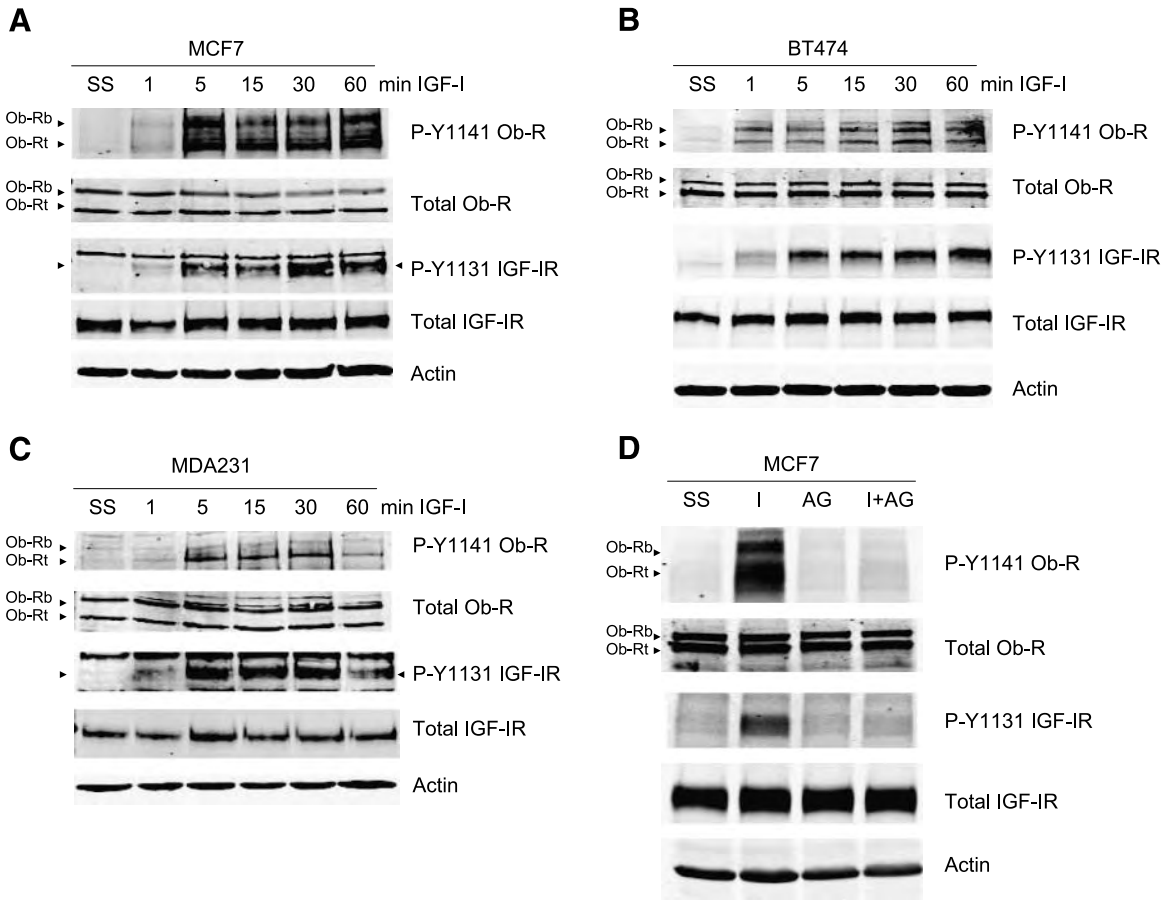

FIGURE 3. Evidence of cross-talk from IGF-IR to Ob-R. IGF-I induces phosphorylation of Ob-R, which is blocked by IGF-IR kinase inhibition. MCF7 (**A**), BT474 (**B**), and MDA231 (**C**) cells were serum-starved overnight and then stimulated with IGF-I (100 ng/mL) for 1, 5, 15, 30, or 60 min. Cells were lysed for protein, and total protein extracts (50 μg) were immunoblotted (SS, serum-starved control) for p-Y^{1141}-Ob-R (phosphorylated Tyr^{1141} on leptin receptor), total Ob-R, p-Tyr^{1131} IGF-IR (phosphorylated Tyr^{1131} on IGF-IR), IGF-IR β, and actin as a loading control. IGF-I stimulated phosphorylation of IGF-IR within 5 min in all cell lines. Importantly, phosphorylation of the leptin receptor was also induced within 5 min of IGF-I exposure. Total receptor levels did not change. **D.** MCF7 cells were serum-starved overnight, then stimulated with IGF-I (100 ng/mL) for 5 min, and/or treated with the IGF-IR kinase inhibitor I-OMe-AG538 (10 μmol/L overnight). Total protein was immunoblotted for p-Y^{1141}-Ob-R, total Ob-R, p-Tyr^{1131} IGF-IR, and total IGF-IR. Experiments were done at least twice. Inhibition of IGF-IR kinase blocked IGF-I–mediated phosphorylation of leptin receptor, supporting cross-talk from the IGF-IR kinase to leptin receptor. SS, serum-starved control; I, IGF-I; AG, I-OMe-AG538; I + AG, IGF-I + I-OMe-AG538.

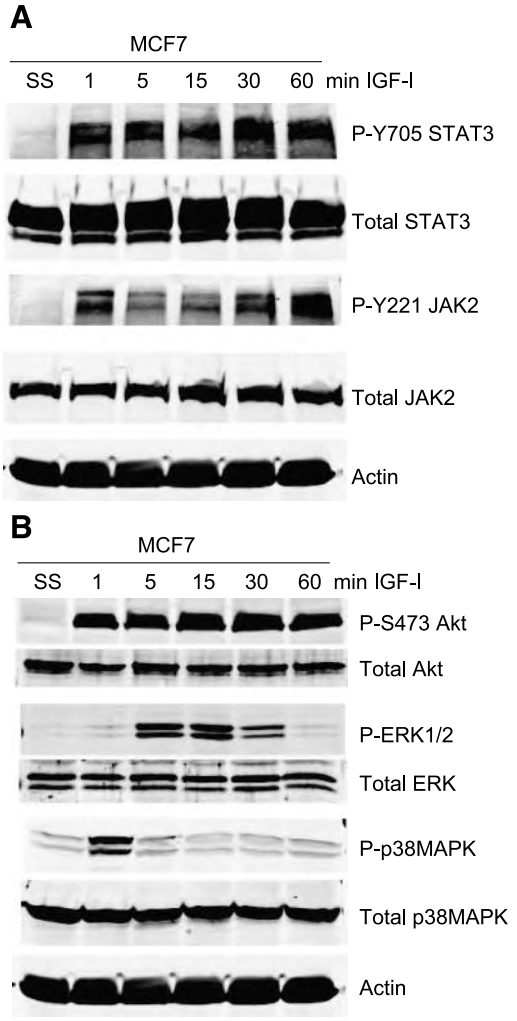

FIGURE 4. IGF-I activates downstream signaling. MCF7 cells were serum-starved overnight and then stimulated with IGF-I (100 ng/mL) for 1, 5, 15, 30, or 60 min. Total protein extracts (50 μg) were immunoblotted for the downstream leptin signaling molecules p-STAT3 (Tyr^{705}), total STAT3, p-JAK2 (Tyr^{221}), and total JAK2 (24^{B11}) (**A**) and for molecules downstream of both leptin receptor and IGF-IR, p-Akt (Ser^{473}), total Akt, p-p42/p44 MAPK (Thr^{202}/Tyr^{204}; ERK1/2), total p42/p44 MAPK (ERK1/2), p-p38 MAPK ($pThr^{180}/Tyr^{182}$), and total p38 MAPK (**B**). IGF-I induced phosphorylation of STAT3 and JAK2, consistent with IGF-I–mediated activation of leptin signaling, and also activated Akt, ERK1/2, and p38 MAPK signaling.

mice were resistant to mammary tumor development (13), illustrating the important contribution of the leptin signaling pathway to some forms of breast cancer. Hence, because IGF-I and leptin are frequently detected in the serum of breast cancer patients and both receptors are overexpressed in a majority of breast tumors, we sought to determine whether molecular interactions occur between IGF-IR and leptin receptor in breast cancer.

We showed the following novel findings (Fig. 6):

(*a*) The IGF-I and leptin receptors interact in human breast cancer cells. Of potential interest, IGF-IR may preferably associate with Ob-Rt versus Ob-Rb in MCF7, BT474, and SKBR3 cells, as more of this isoform was pulled down in the IGF-IR immunoprecipitates (Fig. 2B); total levels of both Ob-R isoforms were similar in each line (Fig. 1A).

(*b*) Cross-signaling occurs from IGF-IR to Ob-R in breast cancer. IGF-I stimulation induces phosphorylation and activation of Ob-R, whereas IGF-IR kinase inhibition blocks IGF-I–mediated Ob-R activation. Downstream signaling molecules examined included JAK2, STAT3, Akt, and ERK1/2, all of which are functional in the leptin and IGF-IR pathways, as well as in multiple other signaling pathways. Thus, the IGF-I signaling experiments do not strictly indicate that IGF-I induces activation of one particular pathway. However, our results clearly indicate that IGF-I induces phosphorylation of Ob-R on Tyr^{1141}. Phosphorylation of Tyr^{1141} is required for Ob-R to bind to the STAT3 transcription factor, which is then activated by JAK2 and translocated to the nucleus to stimulate transcription of downstream target genes (26). Thus, our results indicate that IGF-I activates Ob-R via the IGF-IR kinase.

(*c*) Cross-talk is unidirectional, as leptin does not activate IGF-IR. Whereas it is feasible that other phosphorylation sites on IGF-IR may be affected by leptin stimulation, the three sites examined here (Tyr^{1131} and $Tyr^{1135/1136}$) were not affected by leptin. These three phosphorylation sites are the critical sites known to be required for IGF-IR mitogenicity and transforming activity (27). Thus, the inability of leptin to induce phosphorylation at these sites suggests that the leptin hormone alone is not likely to affect IGF-IR oncogenic function in breast cancer. However, because IGF-IR cross-talks to Ob-R, it is feasible that Ob-R may contribute to IGF-IR molecular or biological effects and is worthy of further study.

Thus, we have identified a novel receptor interaction and unidirectional cross-talk involving the IGF-IR and leptin receptor, which has not been previously described. Interestingly, Garofalo et al. (5) showed that IGF-I can induce leptin transcript levels in MCF7 cells. Our results further support this concept of IGF-I–mediated positive regulation of the leptin pathway.

Cross-talk from IGF-IR to other signaling pathways seems to be a potentially common mechanism used by cancer cells to enhance tumor growth and supports the significance of the IGF-I system to the biology of breast cancer, as well as the relevance of IGF-IR as a therapeutic target. We previously showed that IGF-IR cross-talks to the HER2 cell surface receptor in breast cancer cells that have become resistant to the HER2-targeted agent trastuzumab (19). Others have also shown that IGF-IR is capable of cross-signaling to the EGFR (18) and to the estrogen receptor (14). Thus, understanding the mechanisms by which IGF-IR mediates activation of other growth factor signaling pathways is important to breast cancer research. We have examined the role of the Src kinase family in mediating IGF-IR cross-talk to leptin receptor and have found that Src kinase inhibition does not inhibit IGF-IR/Ob-R cross-talk (not shown). Future studies will examine the molecular mechanisms mediating this receptor cross-talk. In addition, cotargeting leptin receptor and IGF-IR as a strategy to inhibit breast cancer progression, as well as the contribution of leptin receptor to IGF-I–mediated promitogenic and antiapoptotic effects, will be examined in breast cancer cells.

In summary, our results show, for the first time, that the IGF-I and leptin receptors physically form a protein complex in

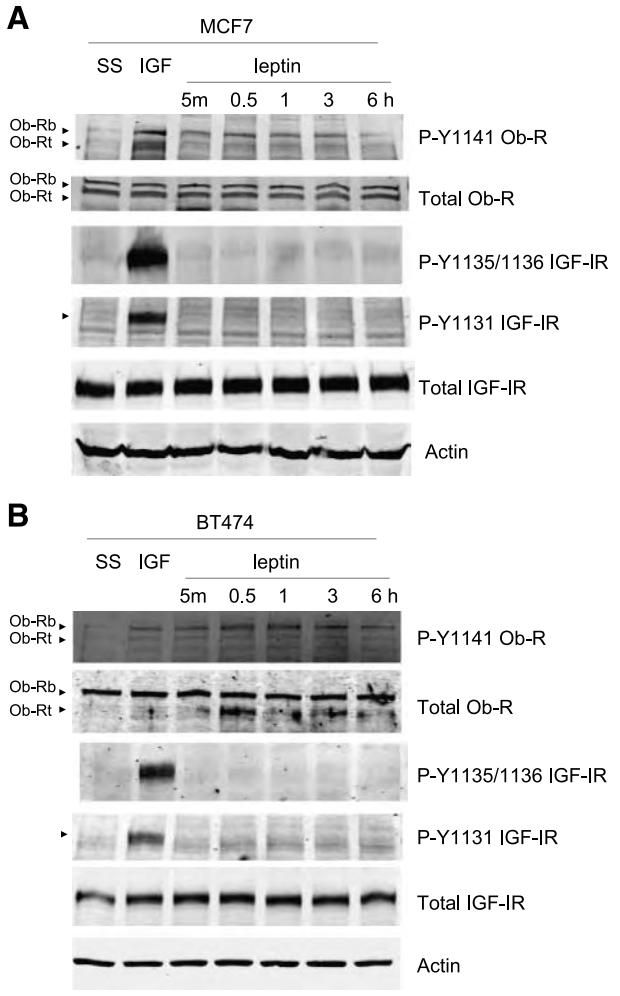

FIGURE 5. Evidence of unidirectional cross-talk. Leptin does not induce phosphorylation of IGF-IR. MCF7 (**A**) and (**B**) BT474 cells were serum-starved overnight and then stimulated with IGF-I (100 ng/mL) for 5 min or leptin (1,000 ng/mL) for 5 min or 0.5, 1, 3, or 6 h. Cells were lysed for protein, and total protein extracts (50 μg) were immunoblotted (SS, serum-starved control) for p-Y^{1141}-Ob-R, total Ob-R, p-Tyr^{1131} IGF-IR, p-$Tyr^{1135/1136}$ IGF-IR, total IGF-IR β, and actin. Experiments were done at least twice. IGF-I stimulated phosphorylation of IGF-IR and Ob-R within 5 min in both cell lines as expected and served as a positive control. Leptin stimulated phosphorylation of Ob-R in both lines but did not induce phosphorylation of IGF-IR at the phosphorylation sites examined, suggesting that receptor cross-talk is unidirectional, occurring from IGF-IR to Ob-R only.

breast cancer cell lines and, further, that there exists a one-way cross-talk whereby IGF-IR induces phosphorylation and activation of the leptin receptor in breast cancer.

Materials and Methods

Materials

Human recombinant IGF-I (Sigma) was dissolved at 100 μg/mL in PBS and used at 100 ng/mL in culture. Human recombinant leptin (EMD Biosciences) was dissolved at 1 mg/mL in PBS and used at 100 or 1,000 ng/mL. I-OMe-AG538 IGF-IR kinase inhibitor (Sigma) was dissolved at 1 mmol/L in PBS and used at 10 μmol/L in culture.

Cell Culture

MDA-MB-231 (MDA231), MCF7, BT474, and SKBR3 breast cancer cells were purchased from the American Type Culture Collection and maintained in DMEM supplemented with 10% FCS.

Ligand Stimulation

Cells were serum starved overnight, and then stimulated with IGF-I (100 ng/mL) for 1, 5, 15, 30, or 60 min or leptin (1,000 ng/mL) for 5 min, 0.5 h, 1 h, 3 h, or 6 h. In addition, a subset of cells were serum starved, treated with the IGF-IR kinase inhibitor I-OMe-AG538 (10 μmol/L overnight), and stimulated with IGF-I (100 ng/mL).

Immunoprecipitation

Total protein lysates (200 μg) were incubated with 1 μg of Ob-R or IGF-IR antibody or 1 μg rabbit IgG, rotating for 4 h, followed by addition of protein A/G-agarose (Cell Signaling) and rotating overnight. Beads were then washed thrice in PBS containing 0.1% Tween 20 and immunoblotted to detect Ob-R (H-300, Santa Cruz), IGF-IR (polyclonal, Cell Signaling), EGFR (monoclonal 1F4, Cell Signaling), or insulin receptor β (polyclonal, Cell Signaling). Blots of immunoprecipitations were quantitated using NIH imaging software ImageJ.

Immunoblotting

Cells were lysed in buffer containing 10 mmol/L Tris (pH 7.5), 100 mmol/L NaCl, 1 mmol/L EDTA, 1% NP40, and protease and phosphatase inhibitor cocktails (Sigma). Total protein extracts (50 μg) were immunoblotted using the following antibodies at the indicated dilutions: IGF-IRβ (polyclonal at 1:1,000; Cell Signaling); p-Tyr^{1131}-IGF-IR/Tyr^{1146}-IR (polyclonal at 1:200; Cell Signaling); p-$Tyr^{1135/1136}$-IGF-IR/$Tyr^{1150/1151}$-IR (polyclonal at 1:200; Cell Signaling); leptin receptor (Ob-R; H-300 polyclonal at 1:200; Santa

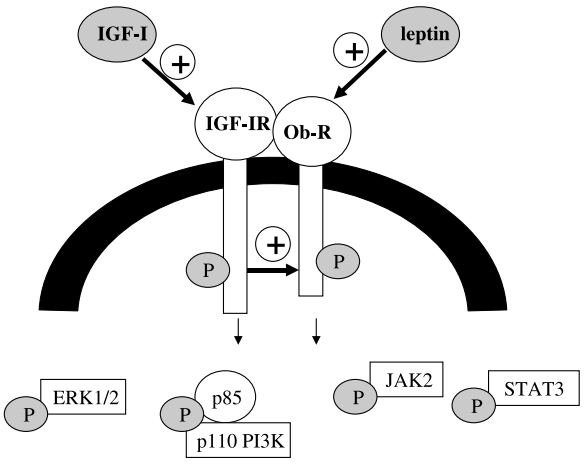

FIGURE 6. A novel unidirectional cross-talk from IGF-IR to Ob-R in breast cancer. Our results indicate that the IGF-I and leptin receptors interact in human breast cancer cells. Furthermore, cross-talk occurs from IGF-IR to Ob-R, such that IGF-I stimulation induces phosphorylation and activation of Ob-R. IGF-IR kinase inhibition blocks IGF-I–mediated Ob-R activation. Cross-talk is unidirectional, as leptin does not activate IGF-IR.

Cruz Biotechnology); p-Y^{1141}-Ob-R (polyclonal at 1:200; Santa Cruz); actin (monoclonal AC-15 at 1:5,000; Sigma Chemical); from Cell Signaling, polyclonal antibodies against p-STAT3 (Tyr^{705}), total STAT3, p-JAK2 (Tyr^{221}), total JAK2 (24B11), total Akt, p-Thr^{202}/Tyr^{204} p42/p44 MAPK (ERK1/2), total p42/p44 MAPK (ERK1/2), p-pThr^{180}/Tyr^{182} p38 MAPK, and total p38 MAPK, monoclonal 587F11 against p-Ser^{473}-Akt, each used at 1:1,000 dilution, and monoclonal 1F4 anti-EGFR used at 1:200 dilution. Secondary antibodies were chosen according to the species of origin of the primary antibody. Protein bands were detected using the Odyssey Imaging System (Li-Cor Biosciences). Bands were quantitated using NIH imaging software ImageJ.

Disclosure of Potential Conflicts of Interest

Q3 No potential conflicts of interest were disclosed.

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